Characterization of Pig-Tailed Macaque Classical MHC Class I Genes: Implications for MHC Evolution and Antigen Presentation in Macaques

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MHC-dependent CD8$^+$ T cell responses have been associated with control of viral replication and slower disease progression during lentiviral infections. Pig-tailed macaques (*Macaca nemestrina*) and rhesus monkeys (*Macaca mulatta*), two nonhuman primate species commonly used to model HIV infection, can exhibit distinct clinical courses after infection with different primate lentiviruses. As an initial step in assessing the role of MHC class I restricted immune responses to these infections, we have cloned and characterized classical MHC class I genes of pig-tailed macaques and have identified 19 MHC class I alleles (Mane) orthologous to rhesus macaque MHC-A, -B, and -I genes. Both Mane-A and Mane-B loci were found to be duplicated, and no MHC-C locus was detected. Pig-tailed and rhesus macaque MHC-A alleles form two groups, as defined by 14 polymorphisms affecting mainly their B peptide-binding pockets. Furthermore, an analysis of multiple pig-tailed monkeys revealed the existence of three MHC-A haplotypes. The distribution of these haplotypes in various Old World monkeys provides new insights about MHC-A evolution in nonhuman primates. An examination of B and F peptide-binding pockets in rhesus and pig-tailed macaques suggests that their MHC-B molecules present few common peptides to their respective CTLs. *The Journal of Immunology*, 2003, 171: 875–885.

MHC class I molecules play a critical role in the recognition of intracellular pathogens (1, 2). They form complexes with β_2-microglobulin and peptides derived from the intracellular degradation of cellular and viral proteins in the endoplasmic reticulum. These stable heterotrimers reach the cell surface and cause, when recognized by the TCR of Ag-specific CD8$^+$ CTLs, the rejection of allogenic cells or the killing of virus-infected cells. MHC class I molecules are encoded by a family of genes, which shares a common organization. They possess eight exons encoding a leader sequence, a peptide binding region (α_1 and α_2 domains), an α_3 domain involved in interactions with CD8 and β_2-microglobulin molecules, a transmembrane region, and a cytoplasmic tail (1, 2). Some MHC class I genes, designated classical MHC class I genes, are highly polymorphic (MHC-A, -B, and -C), whereas others, named nonclassical MHC class I genes, are more conserved (MHC-E, -F, and -G). The variability of classical MHC class I genes is primarily confined to exons 2 and 3, which encode the peptide-binding region, thereby allowing the presentation of a diverse array of peptides. Classical MHC class I and MHC-E molecules are expressed on the surface of nearly all cells. In contrast, MHC-G and -F expression is restricted to specific tissues, with MHC-G molecules limited mainly to trophoblasts, whereas MHC-F is preferentially expressed in lymphoid tissues.

The presence of some MHC alleles has been associated with an increased disease susceptibility or resistance: HLA-B27 confers an increased incidence of ankylosing spondylitis, whereas HLA-B53 is associated with resistance to severe malaria in the Gambian population (3–5). Similarly, several studies have reported a link between MHC class I alleles and disease progression in HIV-1-infected individuals (6). For example, MHC heterozygosity at one or several HLA class I loci is associated with a slower progression to AIDS (7, 8). Some HLA alleles, like HLA-B*57 or B*27, are frequently found in HIV-1-infected long-term nonprogressors, whereas others, like HLA-B*35, are associated with an accelerated clinical course (7, 9–12). Recently, the interaction of some HLA-B molecules with the activating NK cell receptor killer cell inhibitory receptor KIR3DS1 has been associated with delays in progression to AIDS (13). Similarly, in rhesus macaques (*Macaca mulatta*), Mamu-A*01 and Mamu-A*1303 alleles have been associated with reduced viral load and longer survival after SIV or simian-human immunodeficiency virus (SHIV)[2] infections (14–17).

MHC class I genes have been characterized for several primate species including human, the Apes, and New and Old World monkeys (18–31). Studies of rhesus macaque MHC class I genes have led to the development of sensitive immunological assays (e.g., tetramer, ELISPOT, and intracellular cytokine staining) to analyze CD8$^+$ T lymphocyte responses during SIV or SHIV infections in this nonhuman primate (32, 33). In addition to the rhesus monkey, two other macaque species have been used as animal models for HIV-1 infections: the crab-eating or cynomolgus macaque (*Macaca fascicularis*) and the pig-tailed macaque (*Macaca nemestrina*). Cynomolgus and rhesus macaques, both members of the Fascicularis group, are evolutionally related, sharing a common

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[2] Abbreviations used in this paper: SHIV, simian-human immunodeficiency virus; RLM-RACE, RNA ligase-mediated RACE; ORF, open reading frame; UTR, untranslated region.

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ancestor 2.5 million year ago (34). Pig-tailed macaques, a member of the Silenus group, are more divergent but share a common ancestor with the Fascularis group, which entered Asia ~5.5 million years ago (34, 35). Despite a common evolution, macaque species exhibit different sensitivities to primate lentiviral infections: some develop an AIDS-like disease, whereas others remain asymptomatic for several years. Serological studies have been used to characterize the MHC class I genes of pig-tailed macaques (36, 37), but due to their relatively low power of resolution, a rigorous comparison to other macaque species could not be made.

To investigate differences in the immune responses of rhesus and pig-tailed macaques to primate lentivirus infections, we have cloned and analyzed the classical MHC class I genes of the pig-tailed macaque. We found that pig-tailed macaques possess MHC-A, -B, and -I loci. Similar to rhesus monkeys, the MHC-A and B loci are duplicated. The MHC-A alleles of both species form two groups, characterized by 14 polymorphic sites located in exon 2 that form peptide-binding pockets with different properties. Analysis of MHC-A alleles in multiple individual pig-tailed monkeys allowed us to identify three different haplotypes. In addition, an examination of MHC-B allele peptide-binding pockets in rhesus and pig-tailed macaques suggests that each species presents few common peptides to their respective CTLs.

Materials and Methods

Animals

Pig-tailed macaques were wild bred and culled from a colony maintained on an island. After transfer to a National Institutes of Health facility, the animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (38) and were housed in a biosafety level 2 facility. Biosafety level 3 practices were followed. Animals were anesthetized with i.m. injections of ketamine hydrochloride (Ketaject; Phoenix Pharmaceuticals, St. Joseph, MO) and acepromazine acetate (Fermenta Animal Health, Kansas City, MO) during phlebotomies.

Cells

PBMCs were isolated from pig-tailed macaque whole blood using Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation and were activated for 48 h with 5 μg/ml Con A (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin-G, and 100 U/ml streptomycin.

Pig-tailed macaque B cell lines were established by infecting PBMCs, isolated from whole blood using Percoll gradient centrifugation (Amersham Pharmacia Biotech), with *Herpesvirus papio* present in the supernatant of the S-594 cell line, kindly provided by Dr. T. Igarashi (Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The stable B cell lines were maintained in RPMI 1640 medium supplemented with 20% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin-G, and 100 U/ml streptomycin.

Amplification and cloning of pig-tailed macaque MHC class I cDNA

RT-PCR amplification was used for the isolation and cloning of pig-tailed MHC class I genes as described previously (39). Briefly, total cellular RNA was extracted from 5×10^6 activated pig-tailed macaque PBMCs or B cell lines using TRI reagent (Molecular Research Center, Cincinnati, OH). One microgram of this RNA was used to synthesize cDNA with the appropriate reverse primer (Table I) and murine leukemia virus reverse transcriptase. The cDNA synthesis reaction mixture contained 50 mM KCl; 5 mM $MgCl_2$; 10 mM Tris-HCl (pH 8.3); 1 mM each dATP, dTTP, dCTP, and dGTP (GeneAmp; PerkinElmer, Wellesley, MA); 50 pmol of primer; 50 U of reverse transcriptase (PerkinElmer); and 40 U of RNasin (Promega, Madison, WI) in a final volume of 20 μl. cDNA was synthesized at 42°C for 30 min, 95°C for 10 min, and 4°C for 5 min. PCR was then performed with the primers listed in Table I. The PCR mixture contained 50 mM KCl; 1.5 mM $MgCl_2$; 10 mM Tris-HCl (pH 8.3); 1 mM each dATP, dTTP, dCTP, and dGTP; 50 pmol of each primer; and 5 U of AmpliTaq DNA polymerase (Perkin-Elmer). Each reaction contained 20 μl of cDNA in a final volume of 100 μl. The reactions were heated at 92°C for 4 min, and then amplification was conducted for 30 cycles as follows: denature for 1 min at 92°C, anneal for 1 min at 60°C, and extend for 1 min 30 s at 72°C. A final extension was then conducted for 10 min at 72°C.

RNA ligase-mediated RACE (RLM-RACE) was performed using a First Choice RLM-RACE kit (Ambion, Austin, TX) according to the manufacturer's instructions. A single PCR was sufficient to amplify products in 3′ RACE reactions. For 5′ RLM-RACE, a nested PCR was performed using the 5′ RACE inner primer provided by the kit and the A3 MID reverse primer.

PCR products were gel purified using a Geneclean II kit (Bio101, Vista, CA) and then were cloned into the pCR2.1 TOPO cloning vector (Invitrogen, Carlsbad, CA). Individual clones were sequenced using an ABI377 DNA sequencer (Applied Biosystems, Foster City, CA). Following recommendations to avoid PCR-generated mutations for HLA alleles (39, 40), an individual allele was defined by the presence of the identical sequence in at least three independent clones (Table II).

Sequence analysis and phylogeny

Sequences were aligned using the Clustal W program of MacVector 7.1.1 software with minor manual adjustments. Phylogenetic trees were constructed based on the alignment using the neighbor-joining method of the same software (41). Genetic distances were estimated using Kimura's two-parameter method (42). Bootstrap analysis was performed (2000 replicates) to assign confidence to tree nodes (43). Values >70% are shown on the trees.

Nomenclature of primate MHC class I alleles

The names of pig-tailed macaque MHC class I sequences follow the convention recommended by Klein et al. for nonhuman primate MHC alleles, with the first two letters of the genus and species names combined to produce the taxa designation, followed by a hyphen, the letter of the locus type (A, B, C, E, F, or I), an asterisk, and the number of the allele (44). Allele numbers were approved by the Immunogenetics Database/Major

Table I. *Primers used for RT-PCR amplification of MHC class I cDNA from pig-tailed macaques*

Primer Name	Sequence	Location	Sense	Locus
Mane5UB1	AGAGTCTCCTCAGACGCCGA	5′ UTR	Sense	B
Mane5UE	GGAGGCTCTACCGACTCAGA	5′ UTR	Sense	E
Mane5UA	GATTCTCCGCAGACGCCCA	5′ UTR	Sense	A
Mane5	GAACCCTCCTCCTGSTGCTCT	Exon 1	Sense	ABEI
Manex2S	GCTCSCACTCCWTGAGGTATTTC	Exon 2	Sense	ABEI
A3 MID[a]	CCAGGTCAGTGTGATCTCCG	Exon 4	Reverse	ABEI
MamuAex7R	GCACTGTCACTGCTTGCAG	Exon 6/7	Reverse	A
ManeBex7R	GCACTGTCMYTGGACGCAG	Exon 6/7	Reverse	B
ManeEex7R	GYACTGTCGCTACACGMAG	Exon 6/7	Reverse	E
Manex8R	CCACACAAGRCAGYTGTCTCA	Exon 8	Reverse	AB
Mane3UB	TGCCAGAGTGTCTTCAAAAGG	3′ UTR	Reverse	B
Mane3UA	AAGTCAGGGTTCTTCAAGTCA	3′ UTR	Reverse	A

[a] This primer was described by Boyson et al (25).

Table II. *Characteristics of Mane alleles*

	Positive Animals[a]	PCR Reactions[b]	Clones[c]	ORF[d] (aa)	Start[e]	5′ UTR (bp)	3′ UTR (bp)
Mane A							
Mane-A*01	2	8	20	365 F	M1	22	411
Mane-A*02	1	4	6	365 F	M1	2	409
Mane-A*03	3	6	14	365 F	M1	21	413
Mane-A*04	1	2	3	346 p	M1	22	ND
Mane-A*05	1	4	9	351 p	ND	ND	414
Mane-A*06	1	1	4	351 p	ND	ND	376
Mane-A*07	1	1	4	332 p	ND	ND	ND
Mane-A*08	1	4	14	332 p	ND	ND	ND
Mane-A*09	1	1	4	314 p	ND	ND	ND
Mane B							
Mane-B*01	1	1	8	365 F	M1	1	48
Mane-B*02	4	4	30	363 F	M1	10	57
Mane-B*03	2	5	27	359 F	M2	32	58
Mane-B*04	2	4	6	359 F	M2	29	6
Mane-B*05	1	3	8	359 F	M2	30	56
Mane-B*06	1	1	3	349 p	ND	ND	6
Mane-B*07	3	5	12	347 p	ND	ND	6
Mane-B*08	2	3	6	330 p	ND	ND	6
Mane-B*09	3	4	5	330 p	ND	ND	6
Mane I							
Mane-I*0101	1	1	3	333 p	ND	ND	ND

[a] Number of pig-tailed macaques carrying the allele.
[b] Number of PCR reactions in which the allele has been amplified.
[c] Number of individual clones used to define the allele.
[d] Number of amino acid residues encoded by the Mane sequence. Full-length sequences are indicated by F, partial sequences are marked by p.
[e] Alleles begin at either the first ATG codon (M1) or at a second start codon (M2) located 5 bp downstream of M1.

Histocompatibility Complex of Non Human Primates (45). For human MHC class I alleles, the HLA designation was used. The following non-human primate designations were used: gorilla, *Gorilla gorilla* = Gogo; gibbon, *Hylobates lar* = Hyla; rhesus macaque, *M. mulatta* = Mamu; pig-tailed macaque, *M. nemestrina* = Mane; olive baboon, *Papio anubis* = Paan; yellow baboon, *Papio cynocephalus* = *Pacy*; bonobo, *Pan paniscus* = Papa; chimpanzee, *Pan troglodytes* = Patr; and orangutan, *Pongo pygmaeus* = Popy.

GenBank accession numbers of MHC sequences

Mane allele sequences have been deposited in GenBank under accession numbers AY204713–AY204739. The GenBank accession numbers for other sequences used in Fig. 1 are as follows: Gogo-A*0201 (X60259), Gogo-A*0401 (X60257), Gogo-B*0301 (AF157406), Gogo-C*0204 (AF157411), HLA-A*02011 (M84379), HLA-A*2301 (M64742), HLA-A*2422 (AF116214), HLA-B*07021 (M32317), HLA-B*2702 (L38504), HLA-B*4601 (M24033), HLA-Cw*0401101 (M84386), HLA-E*0101 (M20022), HLA-F*0101 (NM 018950), Hyla-A*01 (U50089), Hyla-A*02 (U50090), Hyla-B*01 (U50091), Mamu-A*01 (U50836), Mamu-A*02 (U50837), Mamu-A*03 (U41379), Mamu-A*04 (U41380), Mamu-A*05 (AF161323), Mamu-A*0502 (AF157394), Mamu-A*0504 (AF157396), Mamu-A*06 (U41834), Mamu-A*07 (U41832), Mamu-A*0702 (AF157397), Mamu-A*08 (AF243179), Mamu-A*11 (AF199357), Mamu-A*12 (AF157398), Mamu-A*1303 (AF157401), Mamu-AG*0302 (U84789), Mamu-B*01 (U42837), Mamu-B*02 (U41833), Mamu-B*03 (U41825), Mamu-B*04 (U41826), Mamu-B*05 (U41827), Mamu-B*06 (U41828), Mamu-B*07 (U41829), Mamu-B*08 (U41830), Mamu-B*11 (U41838), Mamu-B*12 (AF243178), Mamu-B*17 (AF199358), Mamu-B*30 (AF157402), Mamu-E*05 (U41837), Mamu-I*01011 (AF161865), Mamu-I*07 (AF161875), Mamu-I*09 (AF161877), Paan-A*01 (U35624), Paan-A*02 (U35625), Paan-A*03 (U35626), Paan-B*01 (U35627), Paan-B*02 (U35628), Pacy-A*01 (AF288698), Pacy-A*02 (AF288699), Pacy-A*03 (AF288700), Pacy-A*04 (AF288701), Pacy-B*01 (AF288702), Pacy-B*02 (AF288703), Pacy-B*03 (AF288704), Pacy-B*04 (AF288705), Pacy-E*01 (AF288706), Papa-B*01 (U05576), Papa-B*03 (U05575), Patr-A*0401 (AF168401), Patr-A*0701 (AF168395), Patr-B*0201 (AF168411), Patr-B*1301 (AF168407), Popy-A*02 (U50084), Popy-A*03 (U50085), Popy-B*0401 (AF118892), Popy-B*0501 (AF118893), and Popy-C*01012 (AF470376).

Results

Cloning of the Mane alleles

Because the sequenced MHC-containing regions of several primate genomes contain multiple MHC pseudogenes, we decided to clone pig-tailed macaque MHC class I genes from mRNA rather than genomic DNA. RNA was extracted from activated pig-tailed macaque PBMCs or from B cell lines established after *Herpesvirus papio* transformation. Consensus primers, capable of recognizing regions flanking exons 2 and 3 of all MHC class I loci of Apes and Old World monkeys, were designed based on GenBank sequences of human (HLA-A, -B, -C, and -E loci), chimpanzee (Patr-A, -B, and -C loci), gorilla (Gogo-A, -B, and -C loci), orangutan (Popy-A and -B loci), gibbon (Hyla-A and -B loci), rhesus macaque (Mamu-A, -B, -I, and -E loci), olive baboon (Paan-A and -B loci), and yellow baboon (Pacy-A, -B, and -E loci) orthologs and are listed in Table I. Exons 2 and 3 of pig-tailed macaque MHC class I genes were initially amplified using the Manex2S and A3 MID primers. The sequences obtained were extended through the 3′ extremities of their open reading frames (ORFs) and into the 3′ untranslated region (UTR) after rapid amplification of their 3′ cDNA ends (3′ RACE). Sequence analysis permitted the design of the type-specific Mane3UA and Mane3UB primers downstream of the ORF stop codon (Table I). The 5′ UTR and 5′ ORF extremities were subsequently determined by 5′ RACE-PCR. Type-specific MHC primers were designed based on the 5′ UTR. Finally, using type-specific MHC primers located in 5′ and 3′ UTR, RT-PCR was used to clone full-length type-A, -B, and -E MHC class I genes.

Using this strategy, 27 pig-tailed macaque MHC class I alleles were cloned and sequenced (Table II). These alleles were compared by phylogenetic analysis with MHC class I genes from Apes (human, chimpanzee, bonobo, gorilla, orangutan, and gibbon) and Cercopithecidae (rhesus macaque and two species of baboon) (Fig. 1). Eight of the alleles grouped

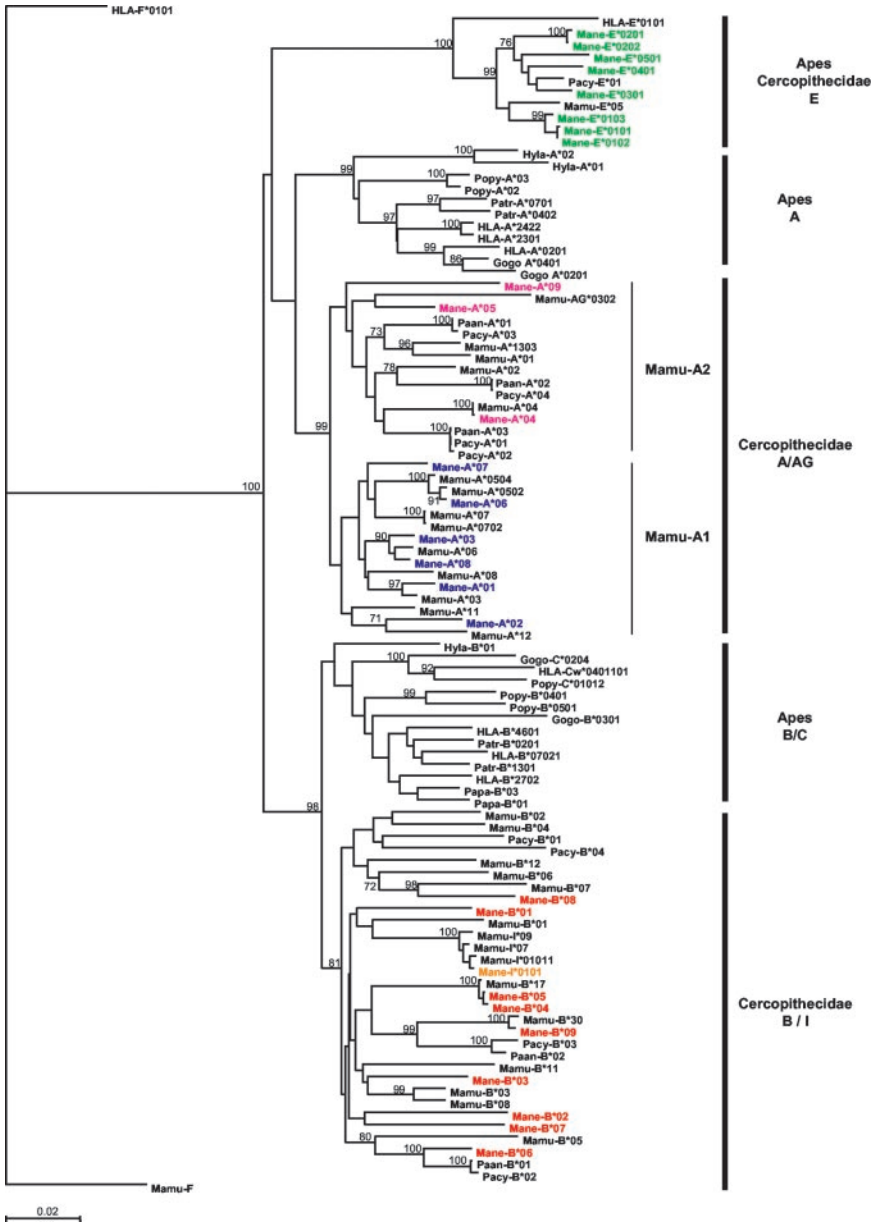


FIGURE 1. Neighbor-joining phylogenetic tree of primate MHC class I coding sequences. Sequence relationship was calculated from a Kimura-2 parameter distance matrix using near full-length MHC class I sequences. Bootstrap values were calculated for 2000 replicates and values >70 are indicated. The primate species examined are human (HLA), chimpanzee (Patr), bonobo (Papa), gorilla (Gogo), orangutan (Popy), gibbon (Hyla), rhesus macaque (Mamu), pig-tailed macaque (Mane), yellow baboon (Pacy), and olive baboon (Paan). The MHC allele names are based on the proposal of Klein et al. (44), with the exception of human (HLA). Mane-E, -B, and -I alleles are shown in green, red, and yellow, respectively. The Mamu-A1 and Mamu-A2 groups of the pig-tailed macaque MHC-A alleles are depicted in blue and pink, respectively.

with MHC-E genes (B. Lafont, A. Buckler-White, R. Plishka, C. Buckler, and M. Martin, manuscript in preparation), nine with MHC-A genes, nine with MHC-B genes, and one with MHC-I genes. The Ape and Cercopithecidae MHC class I genes clustered together for each gene type (A, B/I, and E). However, the Cercopithecidae genes were more closely related to one another, and the alleles from Apes formed a genetically more divergent separate group (with bootstrap values >80). As previously reported (19), MHC-C alleles from Apes were associated with Ape MHC-B genes. Within the Cercopithecidae group, rhesus macaque, pig-tailed macaque, and baboon sequences were interspersed, with no obvious allelic segregation based on species. Pig-tailed macaque (*M. nemestrina*) MHC class I alleles were designated Mane-A, Mane-B, Mane-E, and Mane-I, respectively, following the nomenclature proposed by Klein et al. (44).

All Mane alleles encode proteins having multiple characteristics of MHC molecules (Fig. 2). They contained a leader peptide 18–24 aa in length, two highly variable domains (α1 and 2) involved in peptide and TCR binding, a more conserved domain (α3) involved in interactions with CD8 and β_2-microglobulin, a 38–40 aa transmembrane domain, and a 26–28 aa cytoplasmic tail. An *N*-linked glycosylation site was conserved near the carboxy terminus of the α1 domain, as were four cysteines, two in the α2 domain and two in the α3 domain, involved in intramolecular disulfide bonds.

As expected for classical MHC class I genes, variability of the Mane-A and -B loci was concentrated in exons 2 and 3 encoding the α1 and α2 domains (Fig. 3). Within ORFs, Mane-A and -B contained 156 and 221 polymorphic sites at the nucleotide level, respectively, with 68% and 64% of these changes located in the peptide-binding domains. In contrast, the α3 regions of Mane-A and -B harbored only 15% and 14% of the mutations. The variability of peptide-binding domains was even more marked at the protein level, with 74% and 64% of amino acid changes mapping to α1 and α2 domains, respectively. A majority of the α1 and α2 amino acid substitutions were located at residues predicted to be involved in interactions with either the peptide or the TCR (60% for Mane-A and 55% for Mane-B).

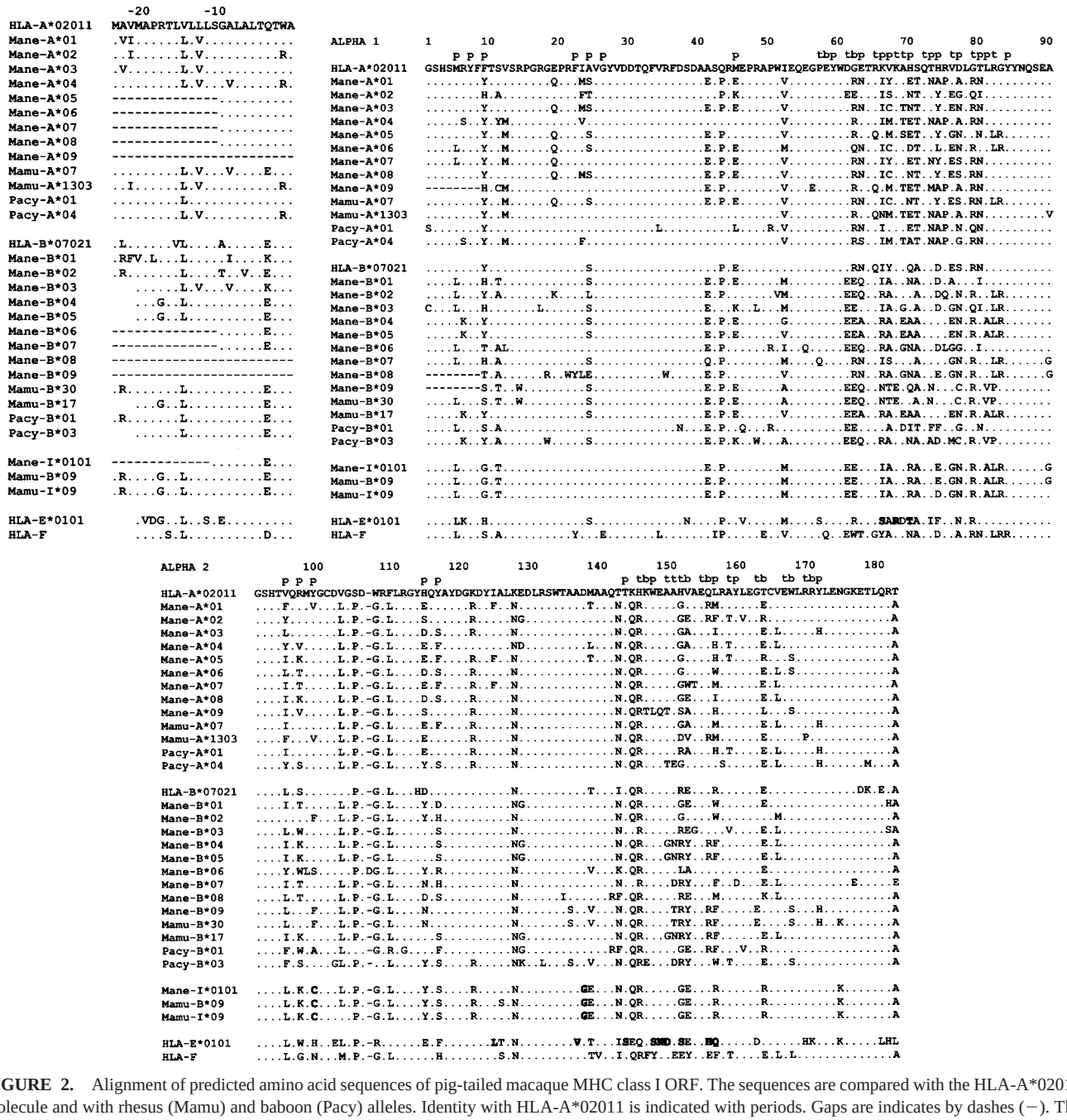


FIGURE 2. Alignment of predicted amino acid sequences of pig-tailed macaque MHC class I ORF. The sequences are compared with the HLA-A*02011 molecule and with rhesus (Mamu) and baboon (Pacy) alleles. Identity with HLA-A*02011 is indicated with periods. Gaps are indicates by dashes (−). The numbering of the mature HLA-A*02011 amino acid sequence is used as reference. Residues that are only found in a single MHC class I type are highlighted in gray. Stop codons are indicated by an asterisk. Residues in the α1 and α2 domains, described in HLA molecules to interact with the peptide, the TCR, or both, are indicated by p, t or b, respectively, below the numbers. (*Figure continues*)

Description of Mane-A alleles

Nine different Mane-A alleles were cloned from five pig-tailed macaques (Table II and Fig. 2); two were present in multiple animals. Full-length Mane-A alleles are 1098 bp long. The ORF starts at the first ATG codon and encodes a 365-aa protein, including the leader peptide. None of the cloned Mane-A alleles were identical with any of the rhesus macaque or baboon MHC-A alleles retrieved from GenBank. The Mane-A*04 allele was closely related to Mamu-A*04 (Fig. 1), containing only a single point mutation that resulted in a valine to alanine change at position 152, a polymorphic residue located in the E pocket of the peptide binding domain in MHC molecules (46). Similarly, Mane-A*06 had three and seven nucleotide changes compared with Mamu-A*0502 and Mamu-A*0504, respectively, resulting in one and three amino acid substitutions, respectively. All of the other Mane-A alleles were more distantly related to rhesus and baboon MHC-A alleles.

Three different Mane-A alleles were identified in four animals, indicating that pig-tailed macaques, like rhesus monkeys and baboons, possess a duplication of the A locus. Phylogenetic analysis, using the neighbor joining method in combination with Kimura-2 parameter distance, divides Cercopithecidae MHC-A class I sequences in two groups (Fig. 1), designated Mamu-A1 and Mamu-A2 by Adams and Parham (20). Both groups contain sequences from rhesus monkey and pig-tailed macaque MHC-A

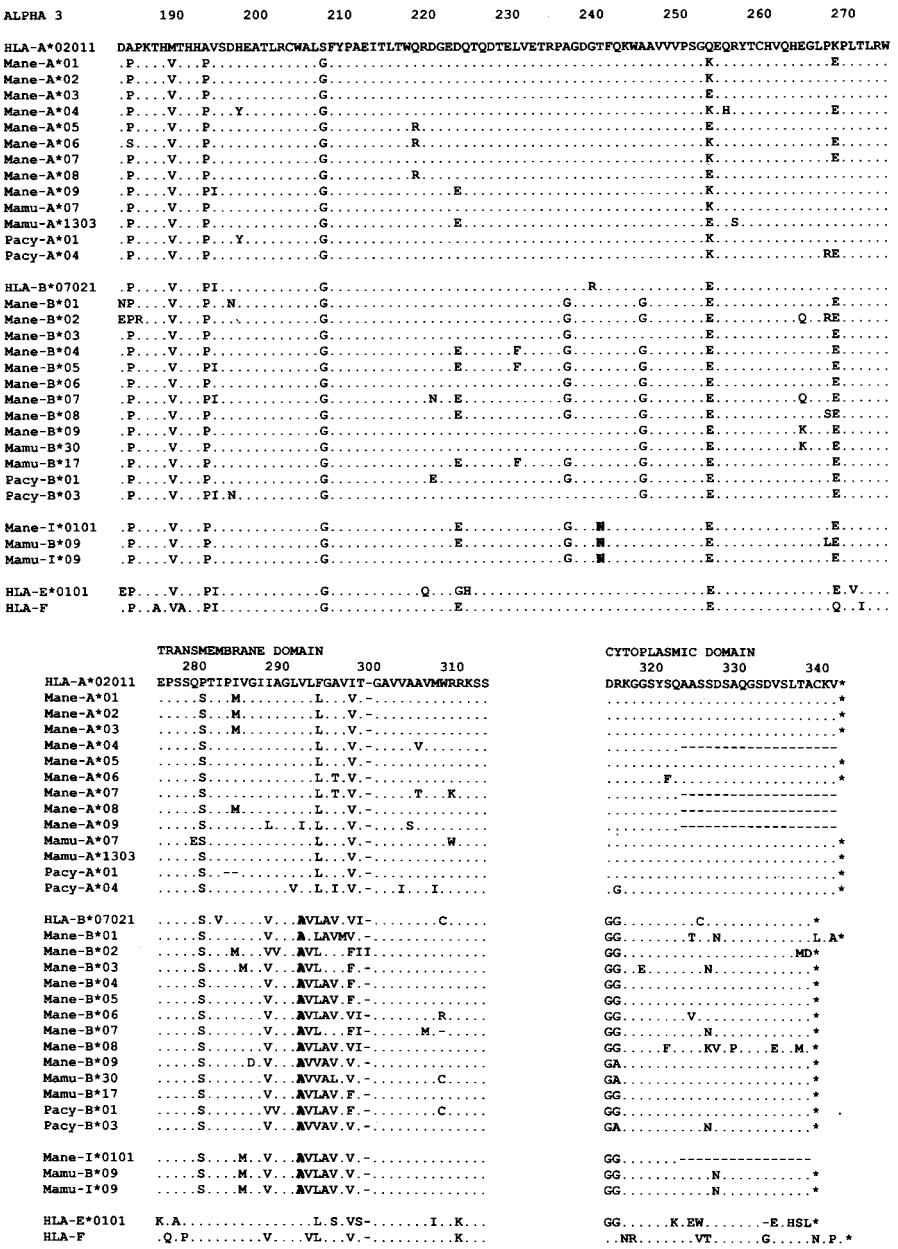


FIGURE 2. *Continued*

class I genes. Baboon MHC-A alleles only contain members of the Mamu-A2 group. We were interested in determining whether these two groups arose as a result of A locus duplication. Among the four macaques from which three Mane-A alleles were cloned, we identified two animals with three Mamu-A1 group sequences. This finding suggests that the Mamu-A1 and Mamu-A2 groups are randomly distributed between the two MHC-A class I loci in individual pig-tailed macaques.

We also investigated which nucleotides might be involved in segregation of Cercopithecidae MHC-A groups. Therefore, additional phylogenetic analyses were performed using the neighbor joining method and Kimura-2 parameter distance on the MHC ORFs, sequentially and alternatively excluding sequences encoding α1, α2, α3, transmembrane, or cytoplasmic domains. Whereas exclusion of α2, α3, transmembrane, or cytoplasmic coding sequences did not affect the segregation into two groups, removal of α1 coding sequences resulted in a collapse of the two MHC-A groups into one. Further analysis of α1 coding sequences revealed that 14 polymorphisms within the second exon were responsible for the MHC-A group division. These changes were located at nucleotide positions 142, 198, 200, 204, 205, 206, 271, 272, 273, 282, 292, 293, 296, and 299 from the start codon (Table III). Nine of these 14 nucleotide substitutions resulted in amino acid changes affecting the B and C peptide-binding pockets. Polymorphisms at nucleotides 205 and 206 were associated with a glutamic acid or a lysine at position 45 in Mamu-A1 group and a methionine or leucine in the Mamu-A2 group. In HLA molecules, a charged residue at position 45 causes a strong selection for a peptide with an amino acid of the opposite polarity at position 2 (2, 47). Conversely, B pockets containing a methionine at position 45 bind peptides having a hydrophobic (A/I/L/V/F) or an uncharged polar (S/T) amino acid at position 2 (2, 47). Therefore, it is quite likely that alleles of Mamu-A1 and Mamu-A2 groups select different peptides based on the amino acid at position 2 (charged in Mamu-A1 group vs hydrophobic or uncharged polar in Mamu-A2). Interestingly, the recent characterization of peptide-binding motifs of some rhesus type-A MHC alleles supports this hypothesis. Mamu-A*01 and Mamu-A*02, both members of the

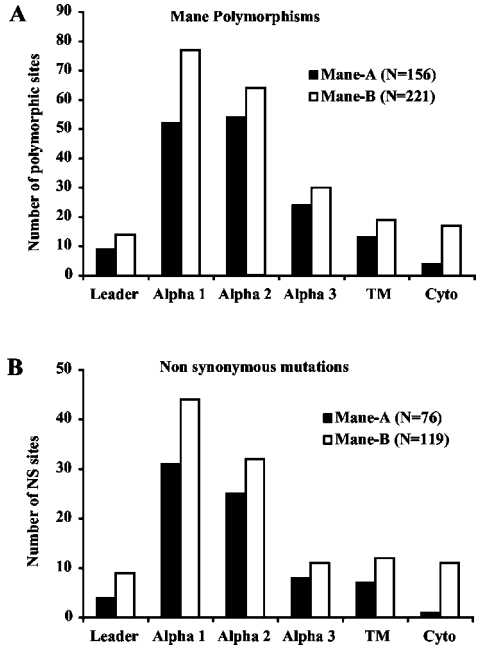


FIGURE 3. Polymorphisms are preferentially located in the α1 and α2 coding sequences of Mane-A and Mane-B alleles. *A*, Total number and location of polymorphic sites were determined for nine Mane-A (156 sites) and nine Mane-B (221 sites). *B*, A similar distribution is shown for nonsynonymous mutations in Mane-A (76) and Mane-B (119) alleles.

Mamu-A2 group, preferentially bind peptides having uncharged polar (S/T) amino acids at position 2 (48–50). Conversely, negatively charged amino acids (D or E) are required at position 2 of the peptide for binding to Mamu-A*11, a member of the Mamu-A1 group (51).

Description of Mane-B alleles

Nine Mane-B alleles were cloned from five different pig-tailed macaques (Table II and Fig. 2). Some of these alleles were also detected in four other animals currently under study. We were able to clone four different Mane-B alleles from each of two animals, indicating that similar to rhesus monkeys and baboons, the B locus of pig-tailed macaques is also duplicated. The full-length size of the Mane-B ORF varied between 1080 and 1098 bp. Similar to Mane-A alleles, some Mane-B ORFs start at the first AUG codon whereas others, due to a deletion of one nucleotide between the two AUGs, begin at a second start site located five nucleotides downstream (Fig. 2). An identical organization has also been reported for some rhesus macaque (Mamu-B*17) and baboon (Pacy-B*03, Pacy-B*04, and Paan-B*02) MHC-B alleles. The Mane-B*06 and Mane-B*02 alleles contain 3-bp insertions between codons 106 and 107 and within the transmembrane coding sequence, respectively. Although neither insertion has been reported for rhesus macaque MHC-B alleles, Mamu-B*04 and Mamu-B*05 both carry 3-bp deletions affecting their transmembrane domains. A mutation of the highly conserved MHC-B allele stop codon, leading to a 3-aa longer cytoplasmic tail in Mane-B*01, is yet another example of ORF length variation.

None of the Mane-B alleles were identical with any of the rhesus or baboon MHC-B alleles in GenBank, but three were closely related to Mamu-B alleles (Fig. 1). The Mane-B*09 allele contained five nonsynonymous mutations compared with Mamu-B*30, changing amino acids at positions 70, 174, 286, 296, and 312. Mane-B*04 and Mane-B*05 alleles differed from Mamu-B*17 by three and two nucleotide mutations, respectively, resulting in a valine to glycine change at position 52 in Mane-B*04 and a valine to isoleucine change at position 194 in Mane-B*05.

In addition, phylogenetic analysis revealed that Mane-B alleles were distinct from Mamu-I alleles, a nonclassical MHC class I locus found in rhesus, cynomolgus, and stump-tailed macaques. A single MHC-I molecule, Mane-I*0101, was cloned from pig-tailed macaques (Figs. 1 and 2). The presence of a type-I MHC allele in the pig-tailed macaque genome indicates that the I locus appeared in the *Macaca* species before the separation of the *Silenus* (including *M. nemestrina*) and *Pro-Fascicularis* (including *M. mulatta*, *M. fascicularis*, and *M. arctoides*) groups. Thus, the I locus, arising from a B locus duplication, was generated before or around the time macaques entered Asia, when the two groups separated ~5.5 million years ago.

Table III. *Mamu-A1 and Mamu-A2 group-specific nucleotides/amino acids*

Nucleotides				Amino Acids		
Nucleotide	Mamu-A1[a] (n = 14)	Mamu-A2[a] (n = 15)	Inversion[b]	Amino acid (pocket)	Mamu-A1[c]	Mamu-A2[c]
142	T/A	G	3/29	24 (B)	S/T	A
198	T	C	4/29	42	S	S
200	C	A	4/29	43	P	Q
204	A	G	1/29	44	R	R
205	G	A/C	3/29	45 (B)	E/K	M/L
206	A	T	1/29	45 (B)		
271	T	A/G	1/29	67 (B)	C/Y, S	M/V
272	G/A, C	T	1/29	67 (B)		
273	C	G	1/29	67 (B)		
282	C	G	5/29	70 (B/C)	N/D	E/A, Q
292	T/C	G	4/29	74 (C)	Y/L	A
293	A/T	C	4/29	74 (C)		
296	G	C/A	3/29	75	R	P/Q
299	A	T/C, G	2/29	76	E	V/A, G

[a] Nucleotides defining the Mamu-A1 and Mamu-A2 groups are listed with the most frequent nucleotide followed by a diagonal and minor nucleotide(s).

[b] Number of alleles of any group having the nucleotide of the alternate group at this position based on 29 Mane-A, Mamu-A, Mamu-AG, Pacy-A, and Paan-A alleles shown in Fig. 1.

[c] Amino acid encoded by the most frequently observed nucleotide. The less frequently encoded amino acids are indicated after the diagonal.

Table IV. *Comparison of B and F peptide-binding pockets from Mane and Mamu alleles*

Locus	Number of Analyzed Alleles	Number of Different Pockets (B/F)	B Pocket Residues[a]											F Pocket Residues[a]									
			7	9	24	25	34	45	63	66	67	70	99	77	80	81	84	95	116	123	143	146	147
Mane-A	9	8/9	Y	YH	STA	V	V	MEK	EN	**IK**	MYCS	EDN	YV	NAGS	NT**I**	L	Y	H	FYS	Y	T	**T**	**L**
Mamu-A	16	10/9	Y	YH	STA	V	V	MEK	EN	I**N**	MYCS	EDN**A**	YV	NAGS	NT	L**A**	Y	H	FYS	Y	T	K	W
Mane-B	9	8/8	Y	YHS**T**	SEA**L**	V	V	M**K**	ENQA	INR	TAS	ANH**Q**	YFS	NDGC	TI	LAV	Y	ILYV	RYS**HD**	Y	T	K	W
Mamu-B	12	12/12	Y	YHS	SEA	V	V**M**	ME	ENQA	INR**K**	TAS**V**	ANH**T**	YFS**H**	NDGC**S**	TI**N**	LAV	Y	ILYV	RYS**F**	Y	T	K	W

[a] Amino acid residues that are unique to a species at a specific position for the same locus are in bold.

MHC-C locus is absent from pig-tailed macaque

We were unable to detect any sequence similar to type-C MHC genes in pig-tailed macaques. Although we cannot exclude the possibility that our PCR primers failed to amplify such MHC genes, even if they were present in pig-tailed macaques, this result obtained is consistent with those reported for rhesus macaque and baboon MHC loci (20, 25, 28).

Peptide binding capability of Mane vs Mamu

To evaluate the peptide binding potential of pig-tailed macaque and rhesus monkey classical MHC molecules, we compared the structure of the B and F pockets, which are known to interact with the most common peptide anchor positions, the second and the carboxyl-terminal residues of the peptide. The amino acid compositions of both pockets were determined for MHC-A and -B alleles in both macaque species (Table IV). Within the limited pool of alleles examined, we noted that the amino acid diversity of both pockets, for each MHC locus, was comparable in the two macaque species. For the B pocket of MHC-A alleles, only two (N and A) and one (K) additional amino acid substitutions were present in rhesus and pig-tailed macaque molecules, respectively. Similarly, one (A) and three (I, T, and L) extra residues were identified in the F pocket of Mamu-A and Mane-A alleles, respectively. The observed number of different B (8 vs 10) and F (9 vs 9) pockets in MHC-A alleles was similar in both macaque species (Table IV). Four of the B pockets and five of the F pockets were identical, representing 28% and 38% of the total B and F pockets present in both species. In contrast, greater amino acid diversity and more unique residues, particularly for the B pockets, were observed for the MHC-B alleles (nine unique amino acids in B pockets and five unique amino acids in F pockets). Consequently, rhesus and pig-tailed macaques share only one common B (5%) and two common F (11%) pockets within their MHC-B molecules.

Discussion

This analysis of pig-tailed macaque classical MHC class I genes revealed that their general organization did not differ significantly from that reported for rhesus macaques (24–26, 52). Indeed, our study has identified several pig-tailed macaque MHC genes orthologous to the Mamu-A and -B loci. The A and B loci are duplicated in both species, and an additional B-like locus, Mane-I, similar to the rhesus nonclassical MHC Mamu-I is also present in the pig-tailed macaque genome. The latter indicates that the MHC-I locus appeared in a macaque ancestor before the separation of rhesus and pig-tailed monkeys 5.5 million years ago. Furthermore, an ortholog of the MHC-C locus was not detected in either macaque species, consistent with the model (19) proposing that the MHC-C locus arose in the Ape lineage after the segregation of the lower Apes (gibbon) from the ancestor of great Apes (orangutan, gorilla, chimpanzee, and human).

A characteristic feature of the macaque MHC class I genes is the duplication of MHC-A loci. Based on phylogenetic analysis of complete primate MHC-A coding sequences, Adams and Parham (20) have described two groups of rhesus macaque MHC-A alleles, which they designate Mamu-A1 and Mamu-A2. Similarly, in their analysis of rhesus macaque MHC class I genes, Boyson et al. (25) have shown that Mamu-A alleles segregate into two groups when their α1 coding sequences are compared with HLA-A and HLA-B sequences. Our study has shown that both MHC-A groups are also present in pig-tailed macaques and can be defined by 14 polymorphic sites located in the α1 domain coding sequence. A majority of these polymorphic sites induce nonconservative amino acid changes affecting the B and C peptide-binding pockets. Therefore, the two phylogenetic MHC-A groups in macaques would appear to possess different peptide-binding characteristics, a hypothesis supported by recent reports describing different binding properties for specific members of each group (48–51).

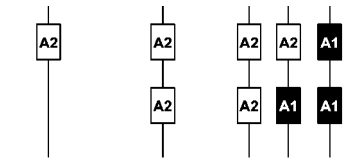
We have found that some individual pig-tailed macaques carried three different Mane-A alleles of the Mamu-A1 group (Table V). Our analysis of previously published rhesus macaque MHC-A genotypes has revealed a similar and unrecognized distribution of three Mamu-A1 or three Mamu-A2 alleles in individual monkeys (Table V) (25, 53). Other animals possessed two MHC-A alleles of each group. These findings suggest the existence of several MHC-A genomic combinations in macaques. If the two MHC-A loci were located on different chromosomes, their distribution

Table V. *Distribution of Mamu-A1 and Mamu-A2 groups in rhesus and pig-tailed macaques*

Species	Alleles			
Pig-tailed macaque[a]	Mane-A			
PT068P	**A*02**[b]	**A*03**	**A*06**	
PT95P028	**A*01**	**A*03**	**A*06**	
Rhesus macaque[c]	Mamu-A			
K556	**A*05**	**A*07**	**A*08**	A*15[d]
84557	**A*05**	**A*06**	**A*07**	
K560	**A*05**	**A*06**	**A*08**	
5615	A*01[e]	A*13	**A*05**	**A*08**
K554	A*02	A*04	A*13	**A*05**
653	A*01	A*02	A*13	

[a] Pig-tailed macaque data are from this study.
[b] Alleles from the Mamu-A1 group are in bold.
[c] Rhesus macaque data are from Boyson et al. (25) and Urvater et al. (53).
[d] Mamu-A*15 could not be assigned to any group because its sequence has not been deposited in GenBank.
[e] Alleles from the Mamu-A2 group are underlined.

within a population would be random. If the two MHC-A loci were present on the same chromosome, they would very likely be distributed in association with one another. Gene duplication is one mechanism thought to be responsible for the polymorphism observed for MHC class I and class II genes, and duplicated genes usually reside on the same chromosome. This also has been observed for several other duplicated cellular genes such as the killer Ig-related receptor genes, the leukocyte Ig-like receptor (LIR/ILT) genes, the CD66 genes, or the D, V, and J segments of the B cell and T cell receptors. Therefore, it would be most surprising if the two macaque MHC-A loci were located on different chromosomes. This suggests that a *cis* configuration of the two loci exists, a condition required for the presence of haplotypes. The existence of an MHC haplotype can be demonstrated: 1) if segregation of polymorphic alleles occurs within a family over several generations, 2) if a homozygous individual results from a consanguineous union, or 3) if a combination of alleles can be detected in several unrelated animals (54). From the distribution of Mamu-A1 and Mamu-A2 groups in rhesus and pig-tailed macaque genomes (Table V), our data fit the third condition. These findings indicate that at least three different MHC-A haplotypes are currently present in the macaque population. One possesses two MHC-A alleles of the Mamu-A1 group, a second has two copies of the Mamu-A2 group, and the third carries one copy of each group (Fig. 4). The presence of several distinct haplotypes in a population is not unique because



Species	MHC-A duplication	A1	A2
Human	no	no	yes
Chimpanzee	no	no	yes
Baboon	yes	no	yes
Macaques	yes	yes	yes

FIGURE 4. Ape and Cercopithecidae MHC-A haplotypes.

humans, chimpanzees, and gorillas possess five, eight, and four different haplotypes, respectively, for MHC-DRB genes (54–56).

Our study also provides information relevant to the evolution of MHC-A genes in baboons. Although yellow and olive baboons each have a duplicated MHC-A locus, only alleles of the Mamu-A2 group have been identified (27, 28). The most likely explanation for this observation is that baboons possess a single haplotype (i.e., two MHC-A genes from the Mamu-A2 group (A2/A2)). This model would predict that the two other haplotypes (A1/A2 and A1/A1) emerged after the separation of the macaque and baboon ancestors, ~10 million years ago. Alternatively, it is possible that the A1/A1 and A1/A2 haplotypes appeared earlier but were later lost from the baboon population. This would be analogous to the reduction of MHC-A lineages in chimpanzees relative to humans (57, 58). It is also possible that, like macaques, baboons possess the two additional MHC-A haplotypes, which have not yet been detected because the sample size currently sequenced is too small. Indeed, only one animal of each baboon species has been analyzed to date, and each carries only alleles from the Mamu-A2 group. A more extensive analysis of the baboon MHC-A alleles will be needed to clarify this issue.

Based on these observations, a model can be proposed to explain the evolution of MHC-A loci in macaques (Fig. 4). A common ancestor of Cercopithecidae and Apes very likely possessed a single Mamu-A2-like MHC-A locus, consistent with the presence in humans and chimpanzees of only MHC-A alleles with characteristic Mamu-A2 codons at residues 24, 43, 45, and 67. It is also worth noting that alleles of the Mamu-A2 group have also been shown to be more closely related to MHC-A alleles of Apes when the α1 coding sequence is used for phylogenic analysis (Ref. 25 and data not shown). In the proposed model, the single Mamu-A2-like MHC-A locus in the progenitor of macaques and baboons underwent duplication, thereby creating two haplotypes: one with a single MHC-A locus and a second with two MHC-A loci. Over time, the duplicated MHC-A haplotype became selected and the single MHC-A haplotype was lost. Alleles with features typical of the Mamu-A1 group emerged in macaques, presumably by gene conversion. In this regard, Boyson et al. (25) have shown that the Mamu-A*03, Mamu-A*05, Mamu-A*06, and Mamu-A*07 alleles, all from the Mamu-A1 group, are more closely related to MHC-B alleles than to MHC-A alleles in their α1 domain coding sequences. A similar association has been observed for all alleles of the Mamu-A1 group when the sequences in Fig. 1 were analyzed using the same region. Thus, the alleles of the Mamu-A1 group may have arisen by gene conversion between one Mamu-A2 locus and a MHC-B locus, rather than by introducing several point mutations into an existing Mamu-A2 allele. In any event, a second gene conversion event is needed to generate the haplotype carrying two alleles of the Mamu-A1 group.

The observed random distribution of the two MHC-A groups in macaques indicates that all three haplotypes have been conserved, and it explains the presence of zero to four alleles of the Mamu-A1 or Mamu-A2 groups in individual contemporary monkeys. Moreover, the conservation of the three haplotypes in both macaque species suggests that the appearance of the Mamu-A1 group of alleles predates the separation of pig-tailed and rhesus macaques. It is possible that the migration from Africa to Asia exposed the ancestor of both species to new pathogens and that the Mamu-A1 group conferred survival advantages. To verify this model, it would be of interest to analyze the MHC-A alleles of the Barbary Ape (*M. sylvanus)*, the only macaque species still living in Africa.

Analysis of the MHC-B locus in humans and chimpanzees has shown that the MHC-B alleles are more variable than the MHC-A alleles. For example, the HLA database in 2002 contains 490

HLA-B allele sequences compared with 250 HLA-A alleles (40). This is also the case for MHC-B alleles of pig-tailed macaques which, compared with Mane-A molecules, contain more polymorphisms and more diversity in the amino acids contributing to peptide-binding pockets (Fig. 2 and Table IV). Moreover, a comparison of B and F pockets between rhesus and pig-tailed macaques supports a different evolution for the B and A loci. Indeed, only limited overlap of B and F pockets was observed between Mamu-B and Mane-B alleles (5% for the B pocket and 11% for the F pocket), whereas the MHC-A alleles of both species shared 28% and 38% of their B and F pocket sequences, respectively. The greater diversity and low number of common peptide-binding pockets in the MHC-B molecules of the two macaques predicts that each species will recognize fewer common peptides.

From an immunological point of view, the potential difference in Ag presentation by pig-tailed and rhesus macaques is interesting because each species can exhibit different clinical outcomes after infection with primate lentiviruses. Some SIV strains are pathogenic in both species, but others induce simian AIDS in pig-tailed macaque and not in rhesus monkeys. For example, pig-tailed macaques have been reported to be more sensitive than rhesus monkeys to SIVagm, a virus that is endemic in multiple subspecies of African green monkeys. They experience a depletion of $CD4^+$ T lymphocytes and develop an AIDS-like disease (59, 60). In contrast, rhesus monkeys are readily infected by SIVagm but, like African green monkeys, fail to develop immunodeficiency. Similarly, chimeric $SHIV_{DH12}$ replicates to high titers and induces an AIDS-like disease in ~30% of infected pig-tailed macaques, but not in rhesus monkeys (61). The differences observed between the two macaque species are most likely due to their ability to control virus replication, particularly during the acute phase of the infection. Thus, understanding the different virologic and clinical outcomes between macaque species may identify critical responses needed to control virus replication and prevent disease development. In this context, we have started to analyze the response of CD8 T lymphocytes in chronically SHIV-infected pig-tailed macaques and to characterize the viral epitopes recognized by the cellular responses.

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